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THE STUDY OF THE ROLE OF CELL POPULATIONS IN REJECTION AND TOLERANCE OF CORNEAL ORTHOTOPICAL TRANSPLANTS

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(Immunomodulatory approaches in corneal allo and xenograft rejection in mouse and rat)

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1. ABSTRACT

Small animal models of orthotopic corneal transplantation offer many advantages for the study of immune mechanisms after grafting - not only because of the similar mechanisms of murine and human corneal transplant rejection but also due to the feasibility of the direct observation of the animal without the need to sacrifice it. The purpose of the thesis was to study this model in mouse and rat. We established allotransplantation (BALB/c to C57BL/6 mouse and Wistar Furth to Lewis rat) and concordant xenotransplantation model (rat to mouse; Lewis to BALB/c and Sprague Dawley to BALB/c) and set up grading schemes for the evaluation of the clinical course after grafting.

Initially, we focused on the effect of the suturing technique on the survival of xenografts and on the efficacy of selected immunosuppressants: cyclosporine A, monoclonal antibody against T cells (anti-Thy-1.2) and AMT (a specific inhibitor of inducible nitric oxide synthase, 2-amino-5.6-dihydro-6-methyl-4-H-1.3-thiazine)). The results demonstrate that the suturing technique significantly affects the outcome of transplantation and, importantly, influences the effectiveness of immunosuppressive regimens and therefore must be taken into account when evaluating the efficacy of immunosuppressive drugs.

FTY720 is a novel immunosuppressant with a completely new mechanism of action. It modifies patterns of T cell migration and sequestration in lymph nodes and the thymus. Our results show that treatment with FTY720, even in monotherapy, substantially delays the inflammatory response in a dose dependent manner after corneal concordant xenotransplantation and prevents the necrosis at the graft margin and sloughing of the xenograft, possibly enabling later graft infiltration. However, we have also found that treatment with FTY720 induces a profound reduction in T and B cell expansion and the expression of B cell activation markers (major histocompatibility complex (MHC) class II and CD86) in draining lymph nodes (DLN) with subsequent impairment of late recruitment of inflammatory cells into the graft. We demonstrate that FTY720, used in isolation, is a potent immunosuppressant in the control of xenogenic corneal graft rejection and shows that it may be possible, at least in experimental settings, to develop long-term acceptance of corneal xenografts.

We also demonstrate that FTY720 is effective in corneal allograft rejection with a potential to reverse rejection even after priming. It limits the early corneal infiltration with CD11c+ cells and prevents both T and B cell expansion in the DLN with subsequent impairment of the late intragraft recruitment of inflammatory cells. FTY720 also selectively decreases cellularity in the DLN of grafted mice on day 20 after transplantation, suggesting that alloantigen-activated dividing cells can be preferentially affected by the FTY720-treatment.

We show that the reduction of MHC class II expression on lymph node B cells can be induced by FTY720 *in vivo* as well as *in vitro*. However, this effect is limited only to lymph node B cells, since CD11c+ or B220+ cells from the spleen are not affected. Although FTY720 restricts MHC class II expression on lymph node B cells, additional stimulation with lipopolysaccharide is capable of partially restoring this expression and thus another pathway involved in MHC class II regulation can to some extent operate in the presence of FTY720. In conclusion, FTY720 reduces levels of MHC class II expression on the surface of lymph node B220+ cells, which may represent an additional mechanism of FTY720-induced immunosuppression.

Corneal graft rejection is mediated mainly by donor-specific CD4+ T cells and the T_H1 response predominates. Redirecting the recipient's immune response from T_H1 towards T_H2 may have a positive effect on the corneal graft outcome. Transduction with AdrIL- 4 (alone or in combination with vIL-10) leads to visible attenuation of the iris vessels reaction shortly after the transplantation as well as during rejection. This happens in contrast to control mice or mice with grafts transduced with Adβ-gal or AdvIL-10 alone. Presumably, this reflects an immunosuppressive effect of IL-4 on the inflammatory reaction in the anterior chamber. *Ex vivo* corneal transduction with AdrIL-4 does not delay corneal graft rejection and, in addition, its application is dose dependently associated with increased corneal opacity. This probably occurs because of eosinophil infiltration induced by eotaxin produced by corneal fibroblasts under the influence of IL-4. Combined treatment of IL-4 and vIL-10 is associated with more pronounced corneal opacity, the increased activity of neovascularization; the combination of IL-4 with low titre of vIL-10 shortens the graft survival. To sum up and conclude, the redirection of the local immune response towards T_H2-type does not suffice to delay corneal allograft rejection. Nevertheless, the signs of immune modulations warrant further research.

Using small animal models in studies of corneal transplantation significantly extends our possibilities to understand immune mechanisms (and not only those associated with transplantation) and also assists in our efforts to uncover the efficacy and possible mechanisms of action of immunomodulatory drugs or approaches. This thesis demonstrates various possibilities these applications provide.

2. ABSTRAKT

Studium imunitních mechanismů souvisejících s ortotopickou transplantací rohovky není možné bez malých zvířecích modelů. Nejenom, že jsou si imunitní mechanismy u myší, potkanů a lidí do určité míry podobné, ale je zde částečně umožněno i jejich přímé pozorování. Cílem této práce bylo studovat model ortotopické transplantace rohovky: zavést model alotransplantace u myši (BALB/c → C57BL/6) a potkana (Wistar Furth → Lewis) a konkordantní xenotransplantační model (z potkana na myš, Lewis → BALB/c and Sprague Dawley → BALB/c). Dále pak bylo cílem zavedení hodnotících kritérií pro sledování klinického průběhu po transplantaci.

Na začátku byl sledován vliv techniky zakončení stehů na přežívání xenotransplantátů a efektivitu vybraných imunosupresivních látek: cyklosporinu A, monoklonální protilátky proti T lymfocytům (anti-Thy-1.2) a AMT (specifický inhibitor inducibilní synthasy oxidu dusnatého (2-amino-5.6-dihydro-6-metyl-4-H-1.3-thiazin)). Naše výsledky demonstrují, že způsob, jakým jsou stehy zakončeny, signifikantně ovlivňuje výsledek transplantace a dále, že má vliv na účinnost jednotlivých imunosupresiv, a proto je třeba tuto skutečnost brát v potaz při hodnocení jejich efektivity.

FTY720 je nová imunosupresivní látka s naprosto novým mechanismem působení. Modifikuje mimo jiné migraci T lymfocytů, jejich sekvestraci v lymfatických uzlinách a tymu. Naše výsledky ukazují, že léčba pomocí FTY720 i v monoterapii, výrazně oddaluje a tlumí v závislosti na dávce zánětlivou odpověď po konkordantní xenotransplantaci rohovky a zabraňuje nekrose okraje transplantátu a jeho odlučování, což pravděpodobně umožní jeho pozdější infiltraci. Současně jsme zjistili, že léčba pomocí FTY720 výrazně redukuje expanzi T a B lymfocytů a expresi aktivačních markerů B lymfocytů (molekul hlavního histokompatibilního komplexu II (MHC II) a CD86) v drénujících lymfatických uzlinách (DLN) s následným snížením infiltrace xenotransplantátu zánětlivými buňkami. Ukazujeme, že FTY720 i v monoterapii silně potlačuje imunitní odpověď po xenotransplantaci rohovky, a že je tak možné, alespoň v experimentální léčbě, dosáhnout dlouhodobějšího přijetí rohovkového xenotransplantátu.

Dále ukazujeme, že FTY720 efektivně brání rejekci rohovkového alotransplantátu a má potenciál zabránit rejekci i při oddáleném podání. Tlumí časnou infiltraci rohovky CD11c+ buňkami, zabraňuje expanzi T a B buněk v DLN a následně interferuje s pozdní infiltrací zánětlivých buněk do transplantátu. FTY720 též selektivně snižuje počet buněk v DLN transplantovaných myší 20. den po transplantaci, což naznačuje, že aloantigenem aktivované dělící se buňky jsou preferenčně ovlivněny FTY720.

Naše výsledky dokládají, že snížení exprese MHC II na B buňkách lymfatických uzlin vlivem FTY720 je možno prokázat v podmínkách *in vivo* i *in vitro*. Nicméně, tento jev je možno pozorovat pouze u B buněk lymfatických uzlin a nikoliv u CD11c+ buněk, či B buněk sleziny. Ačkoliv FTY720 sníží expresi MHC II na B buňkách lymfatických uzlin, stimulace lipopolysacharidem jejich expresi částečně obnoví, což ukazuje, že jiné molekulární cesty účastnící se regulace exprese MHC II mohou

částečně fungovat i v přítomnosti FTY720. Souhrnem lze říci, že FTY720 snižuje expresi MHC II molekul na povrchu B220+ buněk v lymfatických uzlinách, což může představovat další mechanismus imunosupresivního působení FTY720.

Rejekce transplantátu rohovky je zprostředkována především dárcovsky specifickými CD4+ T buňkami a T_H1 typ imunitní odpovědi zde převládá. Posun imunitní odpovědi příjemce směrem od T_H1 k T_H2 by mohl positivně ovlivnit přežívání transplantátů rohovky. Transdukce s AdrIL- 4 (samostatně či v kombinaci s vIL-10) viditelně utlumila reakci duhovkových cév v období krátce po transplantaci i během rejekce na rozdíl od kontrolních myší či myší s rohovkami transdukovanými Adβ-gal či AdvIL-10, což pravděpodobně odráží imunosupresivní působení IL-4 na zánětlivou reakci v přední komoře oka. *Ex vivo* transdukce rohovky s AdrIL-4 neoddálí rejekci alotransplantátu rohovky. Navíc je v závislosti na dávce spojena se zvýšenou opacitou rohovky, pravděpodobně v důsledku infiltrace eosinofilů pod vlivem eotaxinu produkovaného IL-4 ovlivněnými fibroblasty rohovky. Kombinovaná léčba s IL-4 a vIL-10 je doprovázena výraznější opacitou rohovky a zvýšenou aktivitou neovaskularizace a kombinace IL-4 s nízkým titrem vIL-10 zkracuje přežívání transplantátů. Závěrem naší studie je, že posun lokální imunitní odpovědi směrem k typu T_H2 není dostatečně účinný k oddálení rejekce transplantátu rohovky a změny, které jsou v této souvislosti pozorovány, je třeba dále studovat.

Použití malých zvířecích modelů pro studium transplantačních imunitních mechanismů výrazně rozšiřuje naše možnosti porozumět imunitním mechanismům (a to nejen těm souvisejícím s transplantací). Také nám pomáhá v posuzovaní účinnosti imunomodulačních látek či postupů a v rozpoznávání mechanismů jejich působení. Tato disertační práce ukazuje rozmanitost možností, jež tyto aplikace umožňují.

3. PREFACE

In 1985, Williams *et al.* introduced a model of orthotopic corneal transplantation in rat (1) and soon afterwards, in 1990, She *et al.* in mouse (2). Six years later, Hašková *et al.* published their work on the role of major and minor histocompatibility antigens in mouse (3). We obtained a model that is – in comparison with rabbits – more defined, offers inbred and congenic strains and various immunologic reagents and is also less expensive. On the other hand, rat but especially mouse models are technically highly demanding and very challenging.

The fact that the general knowledge of the subject substantially increased on a worldwide scale motivated the extension of the focus of the thesis above and beyond the outlined general aim. This extension was, however, always directed to be closely linked to the main topic of the thesis. Using several new immunomodulatory approaches offered new opportunities in this regard. In addition, the possibility to participate in the work of various domestic and foreign research teams (under the leadership of Prof. Martin Filipec, Assoc. Prof. Vladimír Holáň, Prof. John V. Forrester, Prof. Uwe Pleyer and Dr. Thomas Ritter) enriched the scope of the research, as well as the methodology employed.

4. THESIS OBJECTIVE

The framework aim of the thesis was to study an experimental model of corneal transplantation in mouse and rat with the use of methods of cellular and molecular immunology such as cytology, immunohistology or flow cytometry.

The aims of the thesis may be summarized:

- To establish an experimental corneal transplantation model in mouse and rat and grading schemes for the evaluation of the clinical course after grafting
    - allotransplantation model (mouse to mouse; BALB/c to C57BL/6 and rat to rat; Wistar Furth to Lewis)
    - concordant xenotransplantation model (rat to mouse; Lewis to BALB/c and Sprague Dawley to BALB/c);
- To assess the effect of the suturing technique on the survival of corneal concordant xenografts (Lewis to BALB/c) and the efficacy of selected immunosuppressants (CsA, monoclonal antibody against T cells (mAb anti-Thy-1.2) and AMT (a selective inhibitor of inducible nitric oxide synthase (iNOS));

- To study the efficacy and the mode of action of FTY720 in the corneal concordant xenograft (Sprague Dawley to BALB/c) and allograft (BALB/c to C57BL/6) models;

- To evaluate the clinical efficacy of the IL-4 and vIL-10 adenovirus-mediated *ex vivo* gene transfer in rat corneal allograft transplantation (Wistar Furth to Lewis).

5. INTRODUCTION

For a long time, the cornea together with the brain have been considered to be sequestered from (and ignored by) the immune system because of the absence of lymphatic drainage and because of their existence behind the blood–brain or -ocular barrier. The cornea enjoys the advantage of an immune privilege and there are a number of factors which help to protect it from the immune system (*1*). The major factors contributing to the immune privilege are: avascularity of the cornea; blood ocular barrier; constitutive expression of Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by corneal endothelial cells (*2, 3*); constitutive expression of B7-H1 in the cornea, iris and ciliary body (*4*); low levels of the expression of major histocompatibility complex (MHC) class I molecules on the surface of corneal endothelial cells (*5*); anterior chamber-associated immune deviation (ACAID) and immunosuppressive properties of aqueous humor (*6*).

The importance of the immune privilege seems to be predominantly in avoiding (or rather tapering) the inflammatory response induced by trauma or invading pathogens and in limiting its destructive effect on the visual axis where only a very low threshold for anatomical distortion exists. However, the immune privilege is only relative and despite so many inhibitory mechanisms, corneal grafts are still rejected.

The outcome of human corneal transplantation after one year is generally excellent (about 90% graft survival) (*7, 8*), despite the fact that non-human leukocyte antigens (HLA)-matched grafts are predominantly used. However, the probability for graft survival (normal- and high-risk) is 0.73 at 5 years, 0.62 at 10 years, and 0.55 at 15 years, which may be less than the equivalent rate for some vascularized grafts. This is especially true in cases marked by corneal neovascularization, active inflammation, or a previous history of corneal graft rejection, all considered to be "high-risk" grafts (*7, 9-11*). Notably, there has been no improvement in graft survival over a 15-year time period.

5.1. The immune response - afferent arm

After corneal transplantation, the induction of the immune response includes presentation of corneal antigen to naïve T and B cells, which results in their priming, activation and clonal expansion in draining lymph nodes (DLN) and the spleen. Antigens are transported to secondary lymphoid organs (in addition to the thymus) in a soluble form or by professional antigen presenting cells.

Antigen presentation - direct and indirect allorecognition

The situation in corneal transplantation seems to be different in comparison with solid organ transplantation where direct and, later, indirect allorecognition takes place. In the cornea, the indirect mode of allorecognition mediated by CD4+T cells is accepted as dominant in contrast to other organ transplantations in normal-risk recipients (*12*). When the number of corneal Langerhans cells (LC) present in DLN

is artificially enhanced (either by increasing their number in the graft or if neovascularization/lymphangiogenesis is present), the direct mode of allorecognition significantly contributes (*13*).

5.2. The immune response - efferent arm

Innate immunity is involved from the moment of transplantation. It is induced by the tissue trauma and the following wound healing and works in a close cooperation with corneal cells. Its main activity lasts several days after transplantation (as we know from syngeneic transplantations); later its activity ceases. Clinically, after several days the anterior segment becomes relatively quiet – until the onset of rejection.

There are two routes by which the inflammatory cells can access the graft - either through the recipient cornea or through the anterior chamber after the disruption of the blood-ocular barrier. In syngeneic transplantation, during the first week grafts are infiltrated with a mixture of macrophages and CD4+T cells with their number promptly decreasing after one or two weeks. By the fourth week, they persist in the wound area but not in the center of the graft (*14*). In allografts, the earliest arriving cells within 15 minutes after transplantation are CD11b+MOMA-2+F4/80+ myeloid cells and granulocytes at the limbus and by two hours at the graft interface (plus some F4/80+ cells and neutrophils detected in the graft). The first CD8+ T cells appear in the graft after 16 hours in contrast to CD4+ cells which need two days. In syngeneic transplantation, the early kinetics is similar but with a slower pace. In allografts, the tissue is infiltrated during rejection by numerous macrophages, T cells (again mainly CD4+), natural killer cells (NK) and neutrophils (*15*). The ratio is reversed after three to four weeks post-grafting when CD8+T cells predominate over CD4+ cells (*14, 15*). NK cells were detected during rejection in the epithelial rejection line, the corneal stroma as well as aqueous humor (*14, 15*) and their role is especially dominant in immature recipients.

In high-risk transplantations, grafts are intensively infiltrated especially by macrophages and neutrophils and to some extent also by mast cells with smaller numbers of mast cells and eosinophils detected also in normal-risk allografts (*16*).

In the aqueous humor, CD4+T cells and cells of monocyte/macrophage origin predominate two days after transplantation and the number of T and NK cells further increases later on (*17*). Within the first three days of rejection CD8+ cells predominate over CD4+ cells, while 5-8 days after the beginning of rejection this ratio is reversed (*18*).

The direct depletion/inhibition of individual cell populations may indicate their role, importance or substitutability in the transplant reaction. CD4+T cells are considered the main effector cells in the indirect allorecognition. Their contribution was

experimentally verified by Hašková *et al.* who demonstrated that in normal-risk recipients CD4+ cells are necessary for corneal graft rejection, but not for the rejection of the skin graft (*19*). Similar results were obtained by Yamada *et al.* and in high-risk corneal recipients by Vítová *et al.* (*20, 21*). Their precise role in rejection is still insufficiently understood. They may operate as helper cells in the generation of allospecific effector macrophages (with subsequent release of a variety of soluble mediators) as well as apoptosis-inducing effector cells.

CD8+T lymphocytes are recognized as central to the direct allorecognition but their role in corneal transplantation is not yet completely understood. According to several reports they contribute to the process of rejection but they do not seem to be crucial (*19*). CD8+T cell-mediated cytotoxicity involves local secretion of cytotoxic cytokines (TNF-α and IFN-γ) and apoptosis-inducing mechanisms such as perforin/granzyme, or Fas/FasL pathway.

$\gamma\delta$ T cells represent a small subset of T cells (usually CD4-CD8- but may be also CD8+) and are often associated with various forms of the tolerance induction in other systems but also with ACAID. The blockage of T cell receptor-delta chain and their functional inhibition result in the shorter corneal allograft survival (*22*).

Similarly, through an IL-10-dependent regulatory mechanism, the population of natural killer T (NKT) cells is critical for the development of antigen-specific regulatory T cells in ACAID. In NKT knock out (KO) animals, rejection rate is 100% in contrast with 50% in wild type animals (*23*).

The role of the humoral immune response and the antibody-mediated damage in corneal transplantation remains controversial. In the model of corneal transplantation, the role of B cells is normally considered secondary to T cell responses. B cells are recognized as antibody producing cells, “contributing” to the tissue injury through complement-mediated reactions (*24, 25*).

The cells of monocyte/macrophage origin are recruited to the graft in large numbers. The role of T cells is critical but allograft rejection occurs also in situations when Fas/Fas ligand, perforin or TNF-α pathways are blocked. CD4+T cells function as helper cells in the generation of allospecific effector macrophages (*26*). During the early phase after transplantation, activated macrophages (in addition to neutrophils) contribute to the innate arm of the immune response and the tissue healing by the removal of the dead tissue and by the production of growth factors stimulating fibroblast proliferation, collagen synthesis and angiogenesis. During later stages of rejection, they likely contribute as effector cells in the acquired arm of the immune response. The full extent of this contribution is yet unknown. After activation, macrophages, in response to CD40 signals and IFN-γ, produce various reactive oxygen intermediates, nitric oxide (NO), and lysozomal enzymes. The activation of the inducible form of nitric oxide synthase (iNOS) and the subsequent production of free NO radicals play an important role in anti-infectious and anti-tumor immunity as

reviewed by Korhonen e*t al.* (*27*). During acute allograft rejection activated macrophages expressing iNOS as a T cell-dependent process were detected (*28*). In a mouse model of corneal transplantation, treatment with aminoguanidine, a specific inhibitor of iNOS, significantly delayed allograft rejection (*29*).

Several studies have evaluated DLN response after transplantation; however, these results are difficult to interpret because the percentage of cell populations rather than the total cell numbers were analyzed.

5.3. Xenotransplantation

Despite the fact that corneal grafts are the most frequently transplanted tissue (*8*), there is a continuing shortage of the donor material and corneal xenografts have long been considered a possibility.

Porcine corneas are for many reasons believed to be the most suitable possible source of xenografts in the future. The majority of human anti-pig natural antibodies are directed against one particular carbohydrate determinant - α-galactoside. There is some experimental evidence that porcine corneas lack α-galactoside epitope (except for several keratocytes in the most anterior part). Yamagami *et al.* showed that the corneal concordant xenotransplantation model is characterized by a delayed onset of rejection (opaque grafts at day 6), with strong cellular infiltration only in the recipient cornea (mainly by T cells with contribution of antibody dependent cytotoxicity) and only later on to some extent in grafts (*30*).

The immunoprivileged environment of the cornea appears to provide corneal xenogeneic grafts with some degree of protection, as hyperacute or acute vascular rejection as described for other solid organ transplants does not occur here (*30*). The rejection of corneal transplants is a slower process, which is, however, associated with severe inflammation throughout the entire anterior segment, with necrosis at the graft margin and sloughing of the xenograft. Large numbers of cells are present in the anterior chamber and significant numbers of lymphocytes and cells of the monocyte/macrophage lineage invade the surrounding tissue and, to a lesser extent, the graft (*30, 31*). Corneal xenograft rejection is mediated by CD4+T cells with a minor contribution from complement (*24*). CD8+T cells and NKT cells are not obligatory, but may play a role in rejection in situations where CD4+T cells are absent or their function is impaired (*32*). CD8+T cells were not able to lyse target guinea pig cells *in vitro*, but produced a significant amount of IFN-γ that may mediate this effect (*32*). In addition, recently Tanaka *et al.* showed that eotaxin (a potent eosinophil chemoattractant) is overexpressed during corneal xenograft rejection, and eosinophils represent the majority of infiltrating granulocytes (*33*). Xenograft rejection is associated with the upregulation of T_H1 type cytokine expression (IL-2 and IFN-γ) initially in the recipient cornea and later in the graft (*30*) and with the

increased NO production. T_H2 type cytokines are upregulated but do not correlate with rejection.

Xenografts are characterized by a greater antigenic difference that exist between different species, and therefore the immune response to xenografts is much stronger than to allografts and is difficult to overcome.

* * *

The long years of evolution have provided the cornea with the advantage of the immune privilege but we should always bear in mind its limits. Firstly, the factors involved and the relationships that exist between them must be recognized; secondly, we need to learn how to use those factors for our benefit. When thinking about the immune mechanisms, it is necessary to realize that although the immune reaction starts in the eye, an important part is also played outside – in draining lymph nodes, the spleen and the thymus.

5.4. Immunomodulatory approaches

In clinical practice, topical prophylactic therapy with corticosteroids (prednisolone acetate or dexamethasone) is a standard postoperative treatment in normal- and high-risk patients and is used as treatment of established rejection episodes. Their application typically starts on the day of transplantation. In high-risk situations, in addition to glucocorticoids, immunosuppressants such as CsA, mycophenolate mofetyl or methotrexat are the drugs of choice while the others are used only infrequently (tacrolimus or rapamycin).

Various immunomodulatory approaches were tested in experimental models of transplantation. However, their results may not often be directly applicable to corneal transplantation. Since corneal graft rejection involves mainly T cells and macrophages producing nitric oxide (NO) (*29, 34, 35*), we tested effects of calcineurin inhibitor cyclosporine A (CsA), mAb anti-Thy-1.2 and a specific inhibitor of inducible NO synthase (iNOS) on corneal xenograft rejection in two groups of recipients which differed in the type of the suturing technique used.

The crucial role of DLN in graft rejection and prevention of rejection was shown in both normal- and high-risk models of corneal transplantation (*36-38*), as well as in other models earlier (*39, 40*). However, such approaches require their surgical removal. We decided to test FTY720, a new immunosuppressive drug, which might represent a possible “non-surgical” alternative. FTY720 is a sphingosine analog with a novel mechanism of action. It inhibits the lymphocyte emigration from the peripheral lymphoid organs and the thymus and dramatically reduces the number of lymphocytes, especially T cells in the circulation, grafts and tissues. Sequestration of lymphocytes in lymph nodes and Peyer’s patches (*41*) (although less pronounced in

mice) is believed to be the dominant mode of action of FTY720. Although the mechanisms whereby FTY720 causes lymphopenia are not fully explained, several reports suggest that the downregulation of sphingosine 1-phosphate 1 ($S1P_1$) receptors and their functional inactivation by FTY720 may contribute (*42*). Treatment with FTY720 has a beneficial effect in several other models of organ transplantation, in models of autoimmune diseases and also, for example, in the prevention of the development of pathology in the graft versus host disease. Importantly, despite its strong immunosuppressive potency, its use is associated with limited side effects.

Gene therapy appears to be a promising approach in many fields, including transplantation. Genes of interest (i.e. immunomodulatory) can be applied systemically or locally. Systemic administration enables us to target simultaneously various systems but it may also induce a systemic immunosuppression. Local *ex vivo* genetic manipulation may suppress the ability of the graft to induce rejection, to increase its protective mechanisms and to inhibit anti-graft immune responses. Since corneas can be easily cultured up to four weeks *in vitro*, *ex vivo* genetic manipulation appears to be a promising approach. From the cytokine profile detected in the aqueous humor and within corneal grafts undergoing rejection, it can be concluded that the T_H1 response predominates in corneal graft rejection (*43-45*). T_H2 type cytokines have the capacity to redirect the recipient's immune response towards a T_H2 direction and this modification may have a positive effect on the corneal graft outcome. IL-4 plays a major role in promoting the differentiation of naïve CD4+ T cells into T_H2 type cells and, once they are differentiated, IL-4 functions as their autocrine growth factor. In addition, IL-4 antagonizes the macrophage-activating effects of IFN-γ and suppresses macrophage-dependent reactions. IL-10, produced by T_H2 cells (as well as by other cells), inhibits activation of T_H1 cells, of macrophages and of dendritic cells and it also terminates cell-mediated immune reactions. The EBV-encoded IL-10 homologue (vIL-10) has a prominent anti-inflammatory potential and has significant homology to human and murine IL-10 (*46, 47*). However, it lacks certain T cell stimulatory activities of IL-10 (*48, 49*). In our study, we investigated the efficacy of the combined adenovirus-mediated IL-4 and vIL-10 *ex vivo* gene transfer in a rat model of orthotopic corneal transplantation.

6. SUMMARY OF THE MAIN EXPERIMENTAL RESULTS

6.1. The effect of the suturing technique on corneal xenograft survival

- The use of maximally trimmed suture endings limits the degree of non-specific irritation and significantly delays the onset of rejection (60% prolongation of the mean survival time). It also decreases the induction of neovascularization.
- Treatment with CsA or mAb anti-Thy-1.2 has immunosuppressive effects on corneal allograft survival comparable to the inhibition of iNOS. This observation supports our previous results demonstrating that the activation of iNOS is a T cell-dependent process.
- In contrast with groups of corneal xenograft recipients with long sutures where all tested immunosuppressants (CsA, mAb anti-Thy-1.2 and AMT (a specific inhibitor of iNOS)) significantly prolong raft survival, none of these immunosuppressants shows a similar efficacy in groups of recipients with short sutures.
- In summary, a simple alteration in the suturing technique significantly affects corneal concordant xenograft survival and, more importantly, affects the efficacy of immunosuppressive therapy.

6.2. FTY720 in corneal concordant xenotransplantation

- FTY720, even in monotherapy, substantially delays the inflammatory response associated with corneal xenograft transplantation in a dose dependent manner and prevents the necrosis at the graft margin and sloughing of the xenograft, possibly enabling graft infiltration at the late time point.
- Retention of the xenograft enables the coverage of the donor rat stroma by epithelium of mouse origin. Interestingly, this occurs within 9 days before the graft is clinically rejected. By the fourth week, the coverage is usually complete. At that time, immunohistological studies identify only a small number of mouse-derived epithelial cells migrating across the rat graft in control animals, indicating that reepithelialization was initiated, but is ultimately abortive.
- Treatment with FTY720 induces a profound reduction in T and B cell expansion and expression of B cell activation markers (MHC class II and CD86) with macroscopically visible prevention of the normal increase in the

size of the draining lymph nodes after transplantation and massively reduces cell numbers.

6.3. FTY720 in corneal allograft transplantation

- Monotherapy with FTY720 at a dosage as low as 0.5 mg/kg/d prevents corneal graft rejection. However, the drug is only effective if administered continuously. Clinically, corneas in treated mice are clear at the time of drug withdrawal with only minimal neovascularization on day 20 after transplantation.
- Treatment with FTY720 significantly limits the early infiltration of the grafted eye with CD11c+ cells but has only a little effect on other cell populations. However, 20 days after transplantation, FTY720 considerably reduces all immune cell populations, particularly CD3+ T cells (up to 100 % reduction). These data suggest that FTY720 may selectively limit the early CD11c+ (presumed) DC recruitment to the graft, with probable downstream effects on T and B cell activation in the DLN, and impairment of the allospecific T cell and effector macrophage responses.
- FTY720 markedly restricts the expansion of T and B cells in DLN that is normally triggered by transplantation. The failure of DC to traffic through the graft and transport sufficient levels of alloantigen to the DLN may contribute to reduced T and B cell activation and proliferation in the DLN but other mechanisms may be involved, since postponing the treatment with FTY720 to 6 days after transplantation produces a similar effect.
- FTY720 markedly reduces surface MHC class II expression on lymph node B cells. The fact that the most affected B cell population is also CD40+ may indicate that FTY720 interferes with B cell activation.

6.4. FTY720 affects MHC class II expression on lymph node B cells

- Lymph node B220+ cells cultured in the presence of 50 nM FTY720 or from FTY720 treated mice (0.5 mg/kg intraperitonealy for 48 hours) express markedly reduced levels of MHC class II on their surface. Interestingly, CD11c+ cells do not show any reduction in MHC class II expression similarly to B220+ cells from the spleen.
- Although FTY720 restricts MHC class II expression on lymph node B cells, additional stimulation with LPS can partially restore it. These results provide support to the assumption that the FTY720-mediated effect on B cells´ MHC class II expression may potentially be caused by interference with PKC activation.

6.5. IL-4 and vIL-10 adenovirus-mediated *ex vivo* gene transfer in rat corneal allograft transplantation

- Transduction with AdrIL- 4 (alone or in combination with vIL-10) leads to visible attenuation of the iris vessels reaction shortly after transplantation as well as during rejection in contrast to control mice or mice with grafts transduced with Adβ-gal or AdvIL-10 alone. This probably reflects an immunosuppressive effect of IL-4 on the inflammatory reaction in the anterior chamber.
- *Ex vivo* corneal transduction with AdrIL-4 does not delay corneal graft rejection and, in addition, its application is dose dependently associated with increased corneal opacity. Presumably, this may result from eosinophil infiltration induced by eotaxin produced by corneal fibroblasts under the influence of IL-4.
- Combined treatment of IL-4 and vIL-10 is associated with more pronounced corneal opacity, increased activity of neovascularization and the combination of IL-4 with low titre of vIL-10 shortens graft survival.

7. GENERAL CONCLUSIONS

7.1. The study of the effect of the suturing technique on corneal concordant xenograft survival provides these findings:

- The suturing technique significantly affects the outcome of corneal concordant xenograft transplantation.
- NO is involved in corneal xenograft rejection, but it is not the only mechanism of rejection.
- Importantly, the suturing technique influences the effectiveness of immunosuppressive regimens. This fact must be taken into consideration when evaluating the efficacy of immunosuppressive drugs.
- Our data may therefore suggest that a substantial part of the immunosuppressive effect of selected immunosuppressants subsists in their limiting the contribution of antigen-nonspecific inflammation. Consequently, the dominant effector mechanisms responsible for xenograft rejection are not effectively compromised by this treatment.

7.2. The study of the efficacy and the mode of action of novel immunosuppressive FTY720 in corneal concordant xenotransplantation (rat-to-mouse) demonstrate:

- FTY720 in monotherapy significantly delays the rejection of rat-to-mouse xenogenic concordant corneal transplants in contrast with other xenogenic transplantation models.
- The severely inflamed immunologically rejected xenograft does not support host-derived reepithelialization of the donor xenograft but treatment with FTY720 sufficiently suppresses the severity of the inflammatory response and permits the donor graft epithelial coverage by host cells and/or that FTY720 also promotes epithelial growth and migration. This is probably the first step towards permanent engraftment.
- Treatment with FTY720 induces a profound reduction in T and B cell expansion and the expression of B cell activation markers in draining lymph nodes after transplantation. Since corneal xenograft rejection is not mediated by natural antibodies or CD8+ T cells directly, but rather by CD4+ T cells, the data from these experiments imply that FTY720 mediates its effect via CD4+ T cells.
- In summary, the study shows that FTY720, used in isolation, is a potent immunosuppressant in the control of xenogenic corneal graft rejection in the rat-to-mouse model.

7.3. FTY720 was tested also in the corneal allotransplantation model in mouse and our results demonstrate:

- FTY720 is an effective immunosuppressant in corneal allograft rejection with a potential to reverse rejection even after priming.
- FTY720 limits the early corneal infiltration with CD11c+ cells and prevents both T and B cell expansion in the DLN with subsequent impairment of late recruitment of inflammatory cells into the graft. Additionally, FTY720 appears to have a significant downregulatory effects on MHC class II expression on B cells in draining lymph nodes.

7.4. The effect of FTY720 on MHC class II expression of lymph node cells and splenocytes was tested *in vivo* and *in vitro* and the findings show:

- A significant reduction of MHC class II expression on lymph node B cells can be induced by FTY720 *in vivo* as well as *in vitro*. However, this effect only relates to

lymph node B cells, since CD11c+ or B220+ cells from the spleen are not affected.

- Although FTY720 restricts MHC class II expression on lymph node B cells, additional stimulation with LPS is capable of partially restoring this expression. Therefore, another pathway involved in MHC class II regulation can, to some extent, operate in the presence of FTY720.
- In conclusion, FTY720 reduces levels of MHC class II expression on the surface of lymph node B220+ cells. This may represent an additional mechanism of FTY720-induced immunosuppression.

7.5. The *ex vivo* IL-4 and vIL-10 adenovirus-mediated gene transfer in rat corneal allograft transplantation suggests:

- The redirection of the local immune response towards T_H2-type does not suffice to delay corneal allograft rejection. Nevertheless, the signs of immune modulations warrant further studies.

* * *

Corneal graft rejection represents a serious problem in a number of situations and understanding the immune mechanisms involved in the process is a fascinating task for us. Despite the progress already made, many of the mechanisms are still unknown and, importantly, our results and results obtained by others reveal that the knowledge and evidence acquired from transplantation models of other organs may not be universally applicable to the ocular niche.

Small animal models of corneal transplantation have a very prominent role in our attempts to understand the immune mechanisms (and not only those associated with transplantation) more comprehensively. Also, they help in assessing the efficacy of immunomodulatory drugs or approaches and in uncovering the possible mechanisms of their action. The thesis demonstrates a variety of possibilities these applications provide.

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9. AUTHOR´S PUBLICATIONS

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- **Sedláková K.**, Robertson M., Muckersie E., Duncan L., Filipec M., Forrester JV.: FTY720 Affects MHC Class II Expression on Lymph Node B Cells (submitted)
- **Sedláková K.**, Filipec M., Holáň V.: The effect of the suturing technique on corneal xenograft survival (submitted)
- **Sedláková K.**, Filipec M.: Corneal immunology: a perspective on factors affecting allo- and xenografts (submitted)

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- **Sedláková K.**, Dubská Z.: Choroideremie (case report) – accepted for publication
- **Sedláková K.:** Mřížková dystrofie rohovky (case report) – accepted for publication
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- **Sedláková K.**: Vyšetřovací postupy II - Přední segment
- Fichtl M., Martincová R., **Sedláková K.**, Růžičková E.: Etiopatogeneze a diagnostika glaukomového onemocnění
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- Fichtl M., Martincová R., **Sedláková K.**, Růžičková E.: Terapie glaukomu
- Fichtl M., Martincová R., **Sedláková K.**, Růžičková E.: Glaukomový záchvat
  - *Note.: The authors contributed equally to presented work*